Renewable and Sustainable Energy Reviews 33 (2014) 188–203

Contents lists available at ScienceDirect

Renewable and Sustainable Energy Reviews

journal homepage: www.elsevier.com/locate/rser

Microbial cellulases: Engineering, production and applications

Veeresh Juturu, Jin Chuan Wu*

*Institute of Chemical and Engineering Sciences, Agency for Science, Technology and Research (A*STAR), 1 Pesek Road, Jurong Island, Singapore 627833, Singapore*

A R T I C L E I N F O

Article history:
Received 17 August 2013
Accepted 29 January 2014
Available online 25 February 2014

Keywords:
Cellulase
Lignocellulose
Hydrolysis
Recombinant DNA technology
Application

A B S T R A C T

Depolymerization of cellulose to glucose requires the synergistic action of three key cellulases, endoglucanase (E.C. 3.2.1.4), exoglucanase (E.C. 3.2.1.176) (E.C. 3.2.1.91) and β-glucosidase (E.C. 3.2.1.21). They belong to the glycoside hydrolase (GH) family and catalyze the hydrolysis of glyosidic linkages depolymerizing cellulose to fermentable sugars. Cellulases are naturally produced by a wide spectrum of bacteria and fungi. These enzymes usually exist as cellulosomes attached to the cell wall of bacteria but are secreted into environments in fungi. They exist either as monomers or multimers with each monomer having a simple architecture containing a cellulose binding domain (CBD) and a catalytic domain (CD) interlinked by a linker peptide. Thermophilic, mesophilic and psychrophilic cellulases are quite different in their structures and amino acid compositions. Post-translational modifications such as glycosylation contribute to enzyme function, multiplicity and stability. Recent advances in recombinant DNA technology allow fast identification of novel cellulase genes, large scale production of cellulases and their genetic modifications to make tailor-made enzymes for various applications. Cellulases have been traditionally used in food processing and textile industries. The rapid depletion of fossil fuels and production of cheap fermentable sugars from abundant renewable resources have increased the demand for cellulases in lignocellulose-based biorefinery. However, the lack of a better understanding of the mechanisms of individual cellulases, their synergistic actions and their high prices are the major bottlenecks yet to be overcome for large scale commercial applications of cellulases in lignocellulose-based biorefinery.

Contents

* Corresponding author. Tel.: +65 6796 3803.
E-mail address: wu_jinchuan@ices.a-star.edu.sg (J.C. Wu).

http://dx.doi.org/10.1016/j.rser.2014.01.077
1364-0321

1. Introduction

Cellulose is the major cell-wall polysaccharides of plants. It is entangled with lignin and hemicellulose carbohydrate polymers. Cellulose is a water-insoluble polymer composed of repeated units of β-D-glucopyranose interlinked by β-1,4 glycosidic bonds [1]. In cellulose polymer, each glucan chain length can reach up to 25,000 glucose residues. At the microscopic level 15–45 glucan chains combine to form a microfibril in a regular crystalline arrangement. A group of these microfibrils form a macrofibril or fiber/cellulose fibril or fiber. In its native state cellulose exists as a paracrystalline form with alternative crystalline and amorphous regions. Additionally, in few cases irregularities such as twists or voids exist in the cellulose fibers which increase their total surface area. Due to its crystalline nature cellulose is resistant to degradation [1].

Plant biomass contains significant amount of cellulose which can be exploited as a valuable carbon source for production of value-added chemicals. To attain this task, depolymerization of cellulose into glucose is a prerequisite for microbial fermentation. There are two ways of converting cellulose to glucose: chemical and enzymatic hydrolysis. Chemical hydrolysis is performed using inorganic acids under harsh conditions. The hydrolysates thus obtained contain not only fermentable sugars but also sugar degradation products such as furfural which are toxic to microbes used in successive fermentation steps. Additional detoxification steps are usually needed to remove inhibitors from the hydrolysates [2]. Application of cellulases to hydrolyze cellulose at mild conditions without generating byproducts is more attractive because the enzymatic depolymerization is environmental friendly [3,4]. Here we review the progress in research, development and applications of cellulases with more focus on publications and patents of recent 5 years.

2. Enzyme classification and structures

Three principle cellulases synergistically confer the complete hydrolysis of cellulose. These are endoglucanases (E.C. 3.2.1.4), exoglucanases and β-glucosidases. Endoglucanases hydrolyze glycosidic bonds at the amorphous regions of the cellulose generating long chain oligomers (non-reducing ends) for the action of exoglucanases or cellobiohydrolases, which cleave the long chain oligosaccharides generated by the action of endoglucanases into short chain oligosaccharides. There are two types of exoglucanases, acting uni-directionally on the long chain oligomers either from the reducing (E.C. 3.2.1.176) or non-reducing ends (E.C. 3.2.1.91) liberating cellobiose, which is further hydrolyzed to glucose by β-glucosidases (E.C. 3.2.1.21) (Fig. 1). Recently, another type of cellulases called oxidative type of cellulases has been identified, which depolymerizes cellulose by means of free radical reactions. Along with these three principle cellulases some accessory enzymes such as lytic oxidases act along with the endoglucanases releasing oligomers with reducing ends. In case of *Streptomyces coelicolor,* Cbp21 protein (chitin-binding protein 21) acts as an oxidative enzyme and aids in depolymerization of recalcitrant cellulose [5,6].

2.1. Enzyme classification

Cellulases are grouped into glycoside hydrolases (GH). According to Carbohydrate-Active enZYmes Database (CAZy), endoglucanases are found in the GH families 5–8, 12, 16, 44, 45, 48, 51, 64, 71, 74, 81, 87, 124 and 128. Exoglucanases or cellobiohydrolases are found in GH families 5–7 and 48 and β-glucosidases in GH families 1, 3, 4, 17, 30 and 116 (www.cazy.org).

2.2. Distribution of cellulase genes in bacteria

Cellulases are expressed by a wide spectrum of microbes in nature. The cellulases from bacteria and fungi are described here. In bacteria, cellulases are present as aggregated structures attached to the cells. These extracellular and large enzyme aggregates are known as cellulosomes [7].

To know and assess distribution of cellulase genes and their numbers in various bacterial phyla, genome search was conducted in 1495 bacterial genomes listed in the CAZy database. Out of all 1495 (100%) bacterial genomes only 575 (38%) genomes contain at least a single enzyme involved in the cellulose hydrolysis. Bacteria

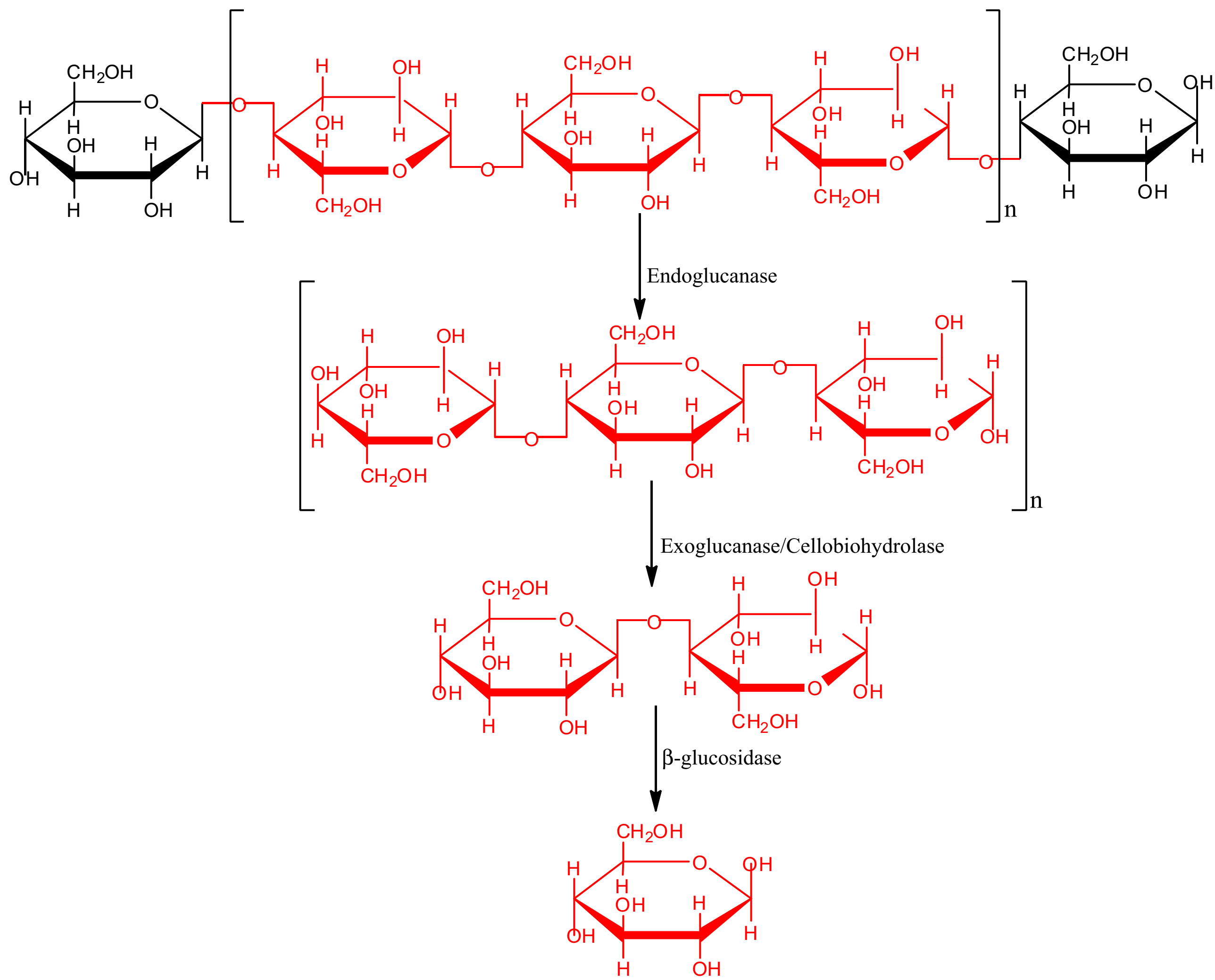


Fig. 1. Structure of cellulose polymer, crystalline cellulose (red color in web/grey in print version) is discriminated from amorphous cellulose (black color) by colors. Principle cellulase sites of action on the cellulose polymer liberating glucose are represented.

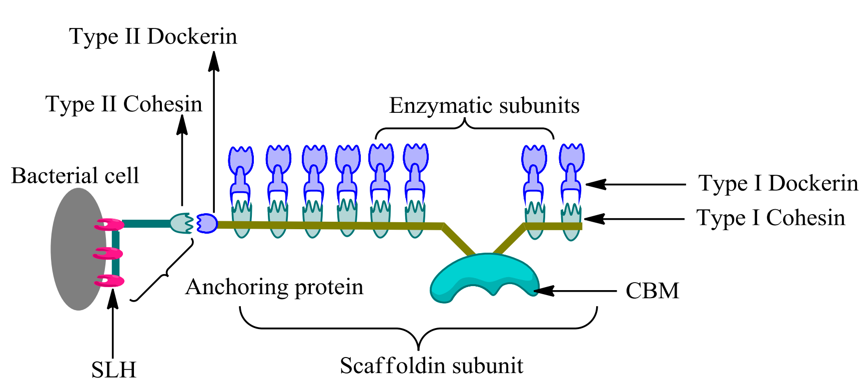


Fig. 2. Schematic architecture of bacterial cellulosome. Cellulosome is attached to the bacterial cell surface by means of anchoring proteins. Cellulosome contains CBM that aides in binding the whole architecture to the substrate. Non-enzymatic scaffoldin protein cohesin provides support to the cellulosomal enzyme subunits. Enzyme subunits bind to cohesin by means of dockerin [7,9].

which contain single cellulase genes have been categorized into four groups. They are saprophytes that do not synthesize cellulose, cellulose-synthesizing saprophytes, cellulose-synthesizing non-saprophytes and those that are neither saprophytic nor cellulose producers [8].

2.3. Cellulosome structure and functions

At microscopic level cellulosomes contain a scaffolding protein on which enzyme subunits are positioned periodically. The scaffolding protein contains cohesins and dockerins (Fig. 2). Each

dockerin at one side binds to the enzyme subunit and another side to the cohesin. Cohesin–dockerin interactions are important in the whole architecture of cellulosomes. Cellulosome compositions are not uniform in all bacterial species. The heterogeneous nature of cellulosomes is due to species specific variation in scaffoldin properties, which allows the assembly and the compositions of cellulosomes to differ among various bacterial species. Most scaffoldins contain 6–9 different cohesins, which can bind up to 26 different cellulosomal enzymes. Depending on the enzyme subunit compositions there is the possibility for making different cellulosomes within a single organism [7].

This unique assembly and architecture of cellulosomes provide multiple advantages which include: (1) a direct and specific adhesion to the substrate of interest permitting efficient competition with other microorganisms present in the same ecological niche; and (2) the proximity of the cell to cellulose insures an efficient cellular uptake of soluble cello-oligosaccharides by avoiding their diffusion into the extracellular space. From the enzymatic viewpoint, cellulosomes (1) allow optimum concerted activity and synergism of the cellulases; (2) avoid non-productive adsorption of the cellulases; (3) avoid unnecessary competition among various cellulases for the sites of adsorption; and (4) allow optimal processivity of cellulases all along the cellulose fiber [9].

2.4. Structure of cellulases

Fungal cellulases have a simple architecture containing a catalytic domain (CD) and a cellulose binding domain (CBD). CD is connected to the CBD by means of a linker peptide. CBD anchors to the cellulose substrate allowing the CD to perform its catalytic function. CBD is not involved in hydrolysis but its removal significantly reduces the enzyme activity towards the substrate [10]. Here the structures of cellulases are explained taking representative enzymes whose structures and functions have been well elucidated in the protein database (www.rcsb.org).

2.4.1. Endoglucanase

The structure of endoglucanase is explained taking the crystal structure of *Thermoascus aurantiacus* endoglucanase (Cel 5A) (PDB ID: 1GZJ). It belongs to GH family 5 (Fig. 3). Upon post-translational modifications 335 amino acid long peptide chain molds into a functional enzyme which has an eight-fold $(\beta/\alpha)_8$ barrel architecture. This enzyme has only one catalytic subunit with a compact structure containing short loops. Enzyme structure has a few extra secondary structures compared to classical eight-fold β/α barrel architecture, which include extra short double stranded antiparallel β sheet in β/α-loop 3 and three one turn helices.

The catalytic glutamates Glu 133 (acid-base) and Glu 240 (nucleophile) are well conserved. Substrate binding site is shallow and long ranging from subsites −4 to +3. This substrate binding site always stays in an active confirmation. A large number of aromatic residues Trp, Tyr and Phe line the groove of the active site covering entire non-reducing to the reducing end of the carbohydrate substrate [11].

2.4.2. Exoglucanase or cellobiohydrolase

The structure of exoglucanases or cellobiohydrolases is explained by taking the crystal structure of *Phanerochaete chrysosporium* cellobiohydrolase (Cel 7A) (PDB ID: 1GPI). This enzyme belongs to GH family 7. After post-translational modifications the 431 amino acid long peptide chain molds into a functional enzyme which has a 3-dimensional β-jelly roll structure (Fig. 4). Dimensions of this β-jelly roll are 62 Å × 41 Å × 48 Å. Each β-jelly roll has two large antiparallel β-sheets facing each other with a hydrophobic interface between these two sheets. Each β-sheet is composed of six strands,

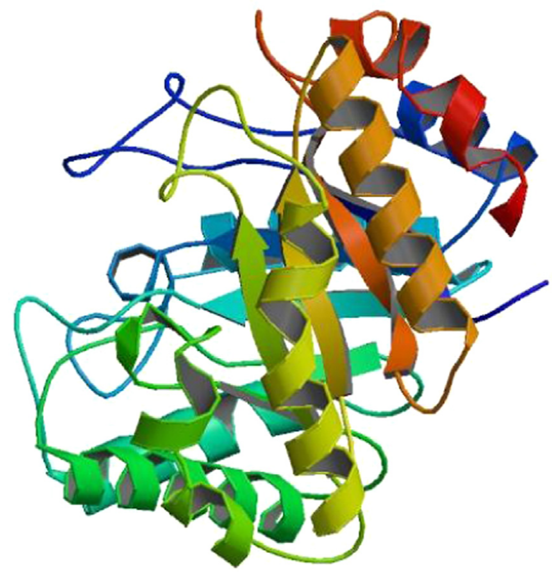


Fig. 3. Crystal structure of *T. aurantiacus* endoglucanase (PDB ID: 1GJH) showing eight-fold $(\beta/\alpha)_8$ barrel architecture which is regular and compact with short loops [11].

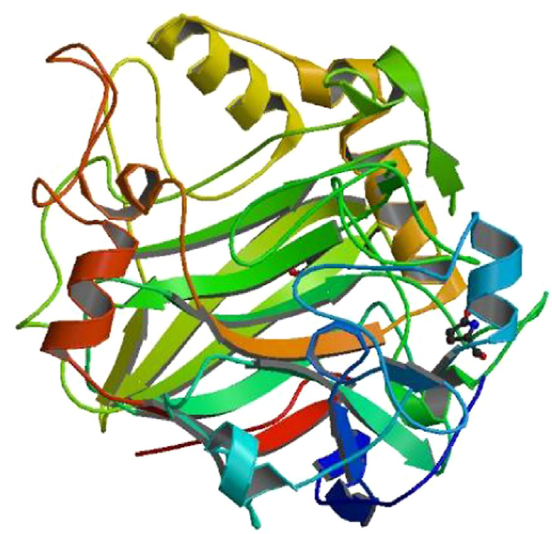


Fig. 4. Structure of *P. chrysosporium* exoglucanase (PDB ID: 1GPI). The protein has a β-jelly roll structure formed by two large antiparallel β-sheets packing one over other [12].

slightly curved developing a convex and concave faces. These β-sheets are interlinked by means of a 4 α-helices. Loops originating from the concave side of the β-sheets are stabilized by nine disulfide bridges. Loops of the β-sheets decide the shape of the substrate binding site. Amino acid residues (427–430) at the C terminus from each monomer interact by means of hydrogen bonding with the same four residues of another monomer creating an antiparallel β-strand interaction. In these interactions Ser 431 takes active participation. To the whole intact protein a linker and CBM connect to the C terminus region of the catalytic domain. Asn 286 is expected to have additional sugar groups which would interfere in the formation of dimer in vivo. N-terminal first amino acid glutamine is cyclised producing a pyroglutamate residue. There are two potential glycosylation sites at Asn 188 and Asn 286. Asn 286 is bound to one *N*-acetyl glucosamine.

Glu 207 is the nucleophile and Glu 212 is the acid/base that donates the proton to the glycosidic oxygen in the first step of the reaction. In the acid/base reaction mechanism, Glu 207 is negatively charged, and Asp 209 would be protonated and

uncharged. Asp 209 is likely to be involved in maintenance of the appropriate pK_a values for the other catalytic residues, assuring the correct charge state of the active site during catalysis [12].

2.4.3. β-Glucosidase

The structure of β-glucosidase is explained by taking the crystal structure of β-glucosidase A from *Bacillus polymyxa* (BglA) (PDB ID: 1BGA). It belongs to GH family 1 and clan GH-A. From the crystallographic data, BglA has an aggregated octameric confirmation with dimensions of 130 Å × 130 Å × 100 Å. In the four-fold axis protein exists as an octamer (Fig. 5). On rotating the protein at 90° reveals close interaction between subunits related by two-fold symmetry generating dimers. Due to this the protein can be described as a tetramer of dimer arranged in a four-fold axis. This may be explained on the basis that native BglA is an intracellular protein, and may exist as a protein aggregate within the heavy traffic of cytosol proteins.

Each monomer adopts a single $(\alpha/\beta)_8$ barrel topology with additional units of secondary structure inserted between α/β subunits. Glu 166 and Glu 352 act as acid/base and nucleophile during hydrolysis reaction, respectively. His 121 is well conserved in GH family 1 and is expected to be involved in substrate binding or transition state stabilization. Tyr 296 is also considered as a potential candidate involved in the catalytic reaction.

Orientation of dimers is very strong in BglA involving 14 H-bonds and water molecules. Helix 7 of the barrel and loop 228–232 make 10 H-bonds, loop 47–50 interact with 441–447 through three H-bonds and Arg 422 side chain links to Lys 365 and Gln 430 [13].

2.4.4. Psychrophilic endoglucanase

The increase in demand for psychrophilic enzymes in biocatalysis paved the way for discovery of psychrophilic cellulases [14,15].

The structure of psychrophilic endoglucanases is explained by taking the crystal structure of the endoglucanase from *Pseudoalteromonas haloplanktis* (PDB ID: 1TVN), which is a Gram negative bacterium isolated from the algae grown in the Antarctica. The endoglucanase from this bacterium is 492 amino acid long, belonging to the GH family 5 and sub-family 2 (Fig. 6). The N-terminal 292 amino acids form the CD, the middle 109 amino acids form the linker region and the C terminal 61 amino acids form the CBD.

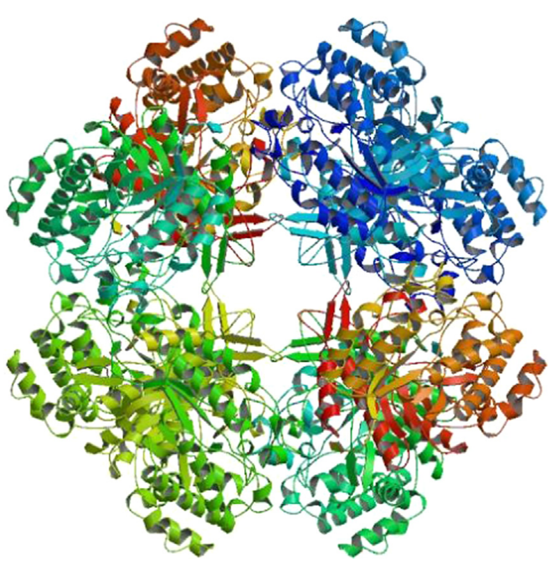


Fig. 5. Crystal structure of β-glucosidase A from *B. polymyxa* (PDB ID: 1BGA) displaying octameric arrangement of enzyme subunits. Closer interactions are found between pairs related by two-fold axes, forming a tetramer of dimers [13].

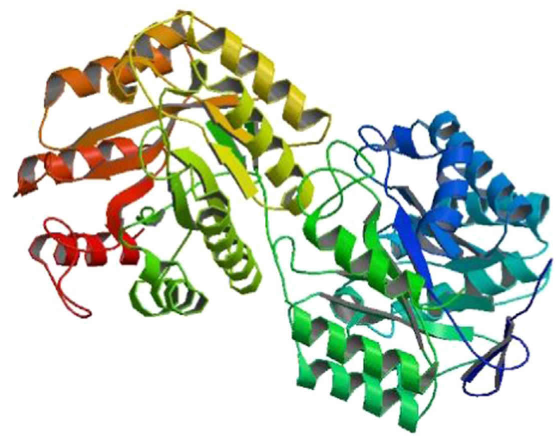


Fig. 6. Side view of the crystal structure of psychrophilic endoglucanase from *P. haloplanktis* (PDB ID: 1TVN) showing both catalytic domain and substrate binding domain. The catalytic module has $(\beta/\alpha)_8$ barrel architecture [14].

The catalytic domain has a classical $(\alpha/\beta)_8$ barrel fold with Glu 135 and 222 acting as a catalytic acid/base [14].

There are few structural modifications in this psychrophilic enzyme that deviate it from the mesophilic cellulases to adapt cold environments. They are: (1) *flexibility*: it is one of the most important properties of the enzyme to show enzyme function at low temperatures. In the CD this flexibility is very high, specifically amino acid residues 227–233 loop connecting β7 with α7. It has been reported that this loop would undergo induced movement on binding to the substrates. (2) *Thermolability*: the flexibility of this enzyme is directly related to the increase in thermolability of the enzyme. Reasons for the thermolability of this enzyme are due to less proline and arginine residues. The increase in the number of glycine residues (up to 21%) and hydrophilic amino acid residues over the CD is supposed to increase flexibility by increasing interaction between the enzyme and solvent. (3) *Electrostatic interaction*: CD contains 52 unequally charged amino acids (34 negatively and 18 positively charged) generating negative potential on the reverse surface of the CD connecting the linker. This electrostatic negative charge repels the linker with a number of positively charged acidic residues thereby the enzyme can adapt to an extended state confirmation on requirement. Further six cysteine residues (Cys 314 and 328, Cys 345 and 359 and Cys 382 and 396) form three disulfide bonds result in three loops each containing 13 residues with five conserved positions. These loops act as spacers generating steric hindrance stabilizing the extended state confirmation without folding into a globular protein in the absence of counter ions [14].

2.4.5. Differences between exoglucanases and endoglucanases

Functionally exo- and endoglucanases perform the same enzyme function, hydrolysis of glyosidic bond. Structurally, the site for cellulose binding is defined by loops. For exoglucanases, they are generally longer and form a tunnel that encloses the catalytic residues. The substrate usually reaches the active site by threading itself from the end of the tunnel. In contrast, endoglucanases have shorter loops that define a more open binding cleft and allow more direct access to intact cellulose chains [16].

2.4.6. Differences between psychrophilic mesophilic and thermophilic cellulases

Psychrophilic and mesophilic cellulases reveal the structural differences and adaptation of proteins to temperatures. The most important structural differences are: (1) cellulases from psychrophilic organisms have high flexibility due to less and/or weaker

intra or inter molecular interactions, (2) less compact hydrophobic packing in the protein core, (3) longer surface loops and fewer proline residues in the loops, (4) increase in the number of glycine clusters, (5) reduced number of proline/arginines, (6) improved solvent interactions by means of additional surface charges (especially negative), (7) increased solvent exposure of a polar surfaces, and (8) a better accessibility to the active site [15].

Similar to psychrophilic cellulases, reasons for the stability of thermophilic cellulases have been well characterized. They are (1) hydrophobic internal protein core, (2) tight compact and rigid structure (high proline content), (3) deleted or reduced loop structures in the protein, (4) high polar surface, (5) fewer and/or smaller voids, (6) small surface area to volume ratio, (7) fewer thermolabile amino acids (Asn, Gln, Met, and Cys), (8) increased H-bonding, (9) high iso-electric point, and (10) more number of salt bridges/ion pairs and networks of salt bridges [17,18].

3. Isolation of cellulase producing microbes

Cellulases are expressed by a wide spectrum of microorganisms in nature. Screening and isolation of cellulase-producing microbes from nature is one of the important ways to get novel cellulases. These newly screened microbes are sources of new cellulase genes with diverse properties. Cellulases are generally produced by saprophytic microorganisms growing on dead and decaying organic matters. Many plant pathogens also express cellulases. Generally, cellulase-producing microbes are isolated from soil samples obtained from forest and nature reserves, hot water springs, compost, sewage, animal manure and bovine rumen. Cellulases from fungi are commercially important as these enzymes are secreted outside the cells and robust. Generally, it is considered that thermophilic cellulases have commercial applications as they are robust and able to resist high temperatures and extreme pH values. However, in recent years cellulases from psychrophilic organisms have received attention. Native cellulase-producing microbes that have been isolated are listed in Table 1. Microbes that surpass commercial conditions such as high levels of cellulase expression and secretion, ability to secrete complete spectrum of cellulases that bring complete depolymerization of cellulose giving high concentrations of sugars (at least 80 g l^{-1}) and cellulases resistant to industrial processes have been patented or even brought into industrial applications. The cellulase genes that have been patented are listed in Table 3.

4. Multiplicity, glycosylation and stability of cellulases

4.1. Multiplicity

Fungal culture supernatants generally contain multiple cellulases which aide in the complete depolymerization of cellulose. It has been recognized in many cases that there is a discrepancy over the expressed cellulases and their coding sequences. Cellulase multiplicity will arise primarily at the level of mRNA (post-transcriptional) due to differential splicing of primary mRNA generating two variants of mRNAs producing two different enzymes. Secondarily, it may arise due to different post-translational modifications of the same protein either by protease cleavage or at the secretory level due to differential glycosylation [19].

Schizophyllum commune can secrete each of all the three principle cellulases. There are two distinct types of cellulases expressed from each mRNA with different molecular weights (exoglucanase 59,300 and 58,200, endoglucanase 40,600 and 39,400, and β-glucosidase 95,700 and 93,800). The cellulases are highly glycosylated. The multiplicity of cellulases arises from both mRNA heterogeneity and differential glycosylation. Heterogeneity at the mRNA level is explained based on multiple initiations of transcription and differential RNA splicing.

Differential glycosylation of the cellulases is explained by the ability of the proteins to bind onto concanavalin-A-agarose during the concanavalin-A fractionation, in which highly glycosylated proteins with high molecular weight are eluted last (70%) indicating the differential glycosylation [19].

Trichoderma reesei is said to secrete extracellular cellulases up to 30 g l^{-1}. However, most of these enzymes occur in multiple forms. It has been argued that the reason for multiple forms of cellulases is proteolysis after secretion. It has been observed that along with low levels of extracellular proteins, cellulase expression also triggers protease expression. The expressed proteases hydrolyze the expressed cellulases yielding both enzyme multiplicity and enzyme specificity towards cellulose [20,21].

Table 1
Native microbial isolates that express cellulases.

Source of microorganisms	Isolated microorganism (s)	Enzyme (s)	Country of origin	Reference
Droppings of elephant	*C. thermocellum* CT2	Cellulosome	India	[98]
Agriculture soil	*Cellulomonas sp. TSU-03*	Cellulosome	Thailand	[99]
Hot water spring	*Anoxybacillus flavithermus, Geobacillus thermodenitrificans, Geobacillus stearothermophilus*	Cellulosome	Egypt	[100]
Salt pans	*Halomonas caseinilytica, Halomonas muralis*	Cellulosome	India	[101]
Compost	*Aspergillus terreus* M11	Endoglucanase, β-glucosidase	China	[102]
Soil near rotten wood	*Fusarium chlamydosporum* HML 0278	Exoglucanase, endoglucanase, β-glucosidase	China	[103]
Soil	*Cellulomonas sp.* YJ5	Endoglucanase	Taiwan	[104]
Gut of silk worm	*Bacillus circulans, Proteus vulgaris, Klebsiella Ineumonia, Escherichia coli, Citrobacter freundii, Serratia liquefaciens, Enterobacter* sp. *Pseudomonas fluorescens, P. aeruginosa, Aeromonas* sp. *Erwinia* sp.	Exoglucanase, endoglucanase	India	[105]
Wood waste from saw mill	*Aspergillus* sp, *Pencillium* sp, *Fusarium* sp, *Botrytis cinerea*	Endoglucanase	Nigeria	[106]
Vinegar waste	*Acetobacter pasteurianus, Acetobacter oboediens, Gluconacetobacter xylinus, Gluconacetobacter hansenii, Gluconacetobacter europaeus, Gluconacetobacter intermedius, Gluconacetobacter entani*	Cellulosome	Turkey	[107]
Persimmon vinegar	*Gluconacetobacter* sp. RKY5, *Gluconacetobacter* intermedius TF2	Cellulosome	Korea	[108]
Empty fruit bunch and palm oil mil effluent compost	*Geobacillus pallidus*	Cellulosome	Malaysia	[109]
Ripe olives	*Cellulomonas flavigena*	Cellulosomes	USA	[110]

4.2. Glycosylation and stability

Cellulases are glycosylated enzymes. Glycosylation of cellulases is not only linked to multiplicity but also to the stability and enzyme activity. Analysis of glycosylation pattern (N- and O-linked) in various cellulases indicated that N-linked glycosylation is the most predominant type present in the CD of the enzyme. The presence of N-glycosylation in the CD often occupies more than half of the peptide length. O-Linked glycosylation is present mainly in the linker region and its flanking regions (~40–45 amino acid residues) closer to the CD. This could be explained by the fact that the linker region is rich in serine thereby providing the sites for the action of serine proteases cleaving apart the CD from CBD. O-Linked glycosylation present on the linker region inhibits the accessibility for serine residues thereby inhibiting the proteases [22].

Cellulomonas fimi is a Gram positive coryneform bacterium. It expresses extracellular cellulases that include exoglucanase (Exg, 46.5 kDa) and endoglucanase A (Eng A, 48 kDa). These native cellulases are glycosylated. On expressing these enzymes heterologously in *Escherichia coli*, enzymes were non-glycosylated but showed all properties similar to native enzymes. Lack of glycosylation does not affect enzyme activity. On exposing to *C. fimi* protease: native enzymes bound to cellulose resisted protease cleavage, whereas non-glycosylated enzymes underwent proteolytic cleavage yielding (Exg, 30 kDa; Eng A, 39 kDa) shorter fragments retaining enzyme activity with reduced affinity to cellulose [23].

Exoglucanase and endoglucanase from *Humicola insolens* YH-8 are glycosylated 26.1% and 39.0%, respectively. By composition, they predominantly contain mannose and N-acetylglucosamine (N-linked glycosylation). These enzymes show remarkable thermal and pH stability. The partial deglycosylation of these enzymes both by means of periodate oxidation and smith degradation significantly reduced their thermal and pH stability without affecting their specific activity [24].

5. Modeling of cellulase kinetics

Enzymatic hydrolysis of cellulose to glucose is one of the prerequisites for successful utilization of this natural biopolymer for production of value-added chemicals. Improving the process of cellulose hydrolysis to yield industrially acceptable glucose levels is always commercially attractive. To achieve this goal many bottlenecks (i.e. feedback inhibition, decrease in enzyme reaction rate at high conversion rates, ratio of principle cellulases, ratio of enzyme to substrate concentration, reaction conditions and reactor design) have to be overcome. Modeling cellulase kinetics is one option to optimize the cellulose hydrolysis [25].

Cellulose hydrolysis requires a robust kinetic model which can accommodate multiple parameters. The two most important factors that significantly affect any model are (1) enzyme and substrate interactions and (2) identification of rate limiting factors [25].

5.1. Kinetics and classification of models

Enzymatic hydrolysis of cellulose involves more steps than classical enzyme kinetics. These major steps include:

1. Binding of cellulases to the substrate via CBD.
2. Recognition of the bond susceptible to hydrolysis by the CD (non-reducing end of oligomer chain in case of cellobiohydrolases/β-glucosidase and internal glycosidic bond in the amorphous region in case of endoglucanase).
3. Formation of enzyme–substrate complex to initiate hydrolysis of cellulose.
4. Hydrolysis of the β-glycosidic bond and simultaneous forward movement of the enzyme along the cellulose chain.
5. Release of cellulase from the substrate or repetition of step 4 or steps 2 and 3 in case if only the catalytic domain detaches from chain.
6. Hydrolysis of cellobiose to glucose by β-glucosidase. Consideration of product inhibition and changes in the substrate properties during the course of hydrolysis,

Based on this fundamental approach all kinetic models till date can be grouped into four classes: (1) empirical models, (2) Michaelis–Menten based models, (3) models accounting for adsorption and (4) models developed for soluble substrates [25].

5.1.1. Empirical models

Empirical models have been generally used to correlate hydrolysis with either the structural properties of the substrate or with time. These models help in (1) understanding the interactions between the substrate properties such as crystallinity, lignin content, or acetyl content and in (2) estimating initial rate reactions important for resuspension experiments and Lineweaver–Burk plots used in the Michaelis–Menten models [25].

5.1.2. Michaelis–Menten based models

Michaelis–Menten models are based on mass action laws that hold true for homogenous reaction conditions and hence cannot be directly applied to the heterogeneous reaction conditions of enzymatic hydrolysis of insoluble cellulosic substrates. The excess substrate-to-enzyme ratio condition which is usually employed for the assumption is not achieved since the fraction of cellulose accessible for adsorption ranges from 0.002 to 0.04. However, Michaelis–Menten models in the literature fit well under the conditions they were developed. Eight different models developed were tested against data of Avicel hydrolysis by *T. reesei* Cel 7A for 24 different substrate-to-enzyme ratios. A model with competitive inhibition by cellobiose was found to fit the data best [25].

5.1.3. Models accounting for adsorption

Incorporation of adsorbed cellulase concentration into modeling system is incorporated by two ways, either Langmuir adsorption isotherm or with the help of kinetic equations. This modeling is based on the assumptions that (1) the adsorption equilibrium is achieved fast when compared to hydrolysis step and (2) the application of the same isotherm at all time points in a reaction assuming that adsorption of enzyme–substrate system does not vary. If both assumptions hold true, then the amount of enzyme adsorbed per unit weight of the substrate can only increase [25].

5.1.4. Models on soluble cello-oligosaccharides

Very limited models have been published on the cellulase hydrolysis of soluble cello-oligosaccharides. These models explain the effects of cello-oligosaccharides on enzyme activity. For example they help in revealing hydrolysis patterns of *T. reesei* Cel6A and Cel 7A. Binding the constants of these enzymes with cello-oligosaccharides increases up to cello hexasaccharide and remains constant for hepta and octasaccharides providing information on span of active site. However, models developed on the soluble substrates cannot be extrapolated to insoluble substrates. This is due to heterogenous action of cellulases over insoluble cellulose [25].

6. Over-expression of cellulases and activities

One of the important prerequisites for enzymatic depolymerization of cellulose is the application of large volumes of enzymes

in the bioprocesses. Cost-effective production of these enzymes in large scales is one of the bottlenecks that have to be overcome. Over-expression of individual cellulases using recombinant DNA technology is the routinely used method [2].

6.1. Expression in bacteria

E. coli and *Bacillus* are the most commonly used platform bacteria for expressing the recombinant proteins. The advantages of using these systems have already been addressed previously [2]. In addition to these two bacteria, a few other expression platforms including *Zymomonas mobilis, Pseudomonas cellulosa* and *Streptomyces lividans* are worth considering for expressing cellulases.

Z. mobilis is a Gram negative, anaerobic and fermentative bacterium. It ferments a wide variety of fermentable sugars to ethanol, yielding ethanol at high concentrations with high tolerance to the toxicity of ethanol [26]. Because of its superior properties, it is considered as an alternative to yeast in alcohol production. The advantages of using this bacterium as a platform for recombinant protein expression include (1) approximately 12–30 times higher protein expression yields compared to *E. coli* [27]; (2) possibility to express the protein both extracellularly and intracellularly [26,28]; (3) relatively simple transformation techniques; (4) possibility for keeping the expression plasmid both as a autonomous replicating DNA or integrating onto its genome [27]; and (5) proven high level expression and secretion of cellulases [26]. The disadvantage is the lack of availability of a commercial kit at this moment.

Pseudomonas cellulosa is a Gram negative, non-cellulosomic saprophytic bacterium. Its genomic DNA contains a complete set of cellulase and hemicellulase genes required for the hydrolysis of plant biomass (http://hamap.expasy.org/proteomes/CELJU.html). Native 23 kDa cellulase gene from this organism was cloned into a low copy number plasmid pMMB66. Recombinant plasmids generated were transformed into host strain *Pseudomonas cellulosa* ATCC 55702. Evaluation of cellulase expression by transformants at colony levels was done using Congo red assay by growing the cells for 24 h in the medium containing CMC (carboxy methyl cellulose). Zones of decolorization were much bigger (11 mm) around the colonies when compared to the host cells without plasmids (4 mm), indicating a four-fold improvement in the expression level of cellulases. This was the first report on recombinant protein expression in this host [29].

Streptomyces lividans is a Gram positive soil bacterium well known for its production of secondary metabolites. This bacterium is reputed as a host for production of recombinant proteins [30]. Advantages of using *Streptomyces* as a host for recombinant protein expression are (1) proven track record for expressing biopharmaceutical proteins and well established protocols for large scale expression of proteins; (2) ability to secrete recombinant proteins; (3) relatively simple protocols required for bacterial transformation; and (4) possibility for engineering the cells for improving recombinant protein expression [31,32].

6.2. Expression in yeast

Yeasts are the most commonly used expression platforms alternative to bacterial expression systems. Advantages of using yeast as a host for recombinant protein expression include: (1) ability to perform eukaryotic post-translational modifications; (2) ability to secrete recombinant proteins; (3) ability to grow to very high cell densities; (4) availability of a wide variety of yeast strains for recombinant protein expression; and (5) yeast cells being relatively free of toxins. The most commonly employed yeast strains for recombinant protein expression are *Saccharomyces cerevisiae, Pichia pastoris* (*Komagataella pastoris*)*, Hansenula polymorpha, Kluyveromyces lactis and Yarrowia lipolytica* [2].

6.3. Expression in filamentous fungi

Many filamentous fungi secrete multiple enzymes. For example, *Aspergillus niger* is capable of producing 25–30 g l^{-1} of glucoamylase and *T. reesei* can secrete 100 g l^{-1} of extracellular proteins. Owing to their high protein secretion capabilities fungi would be an ideal platform for heterologous expression of cellulases using recombinant DNA technology [33]. Most commonly used fungal strains for recombinant protein expression are *Aspergillus nidulans*, *A. niger*, *Aspergillus oryzae*, *T. reesei*, *Penicillium chrysogenum* and *Rhizopus oryzae*. There are few strategies for further improving the titers of recombinant proteins, including (1) *gene fusion strategy*: fusing the nucleotide sequence of protein of interest to the C-terminus of the native secreted protein gene. After expression, the protein of interest can be obtained by protease cleavage; (2) *over-expression of chaperones*: during recombinant protein expression, improperly folded proteins activate protease stress responses cleaving the recombinant proteins. Over-expression of chaperones that assist protein folding would improve secretion levels of recombinant proteins; (3) screening for multi-copy strains. After fungal transformation, isolation of clones with multiple copies (5–6) of recombinant plasmids would improve the recombinant protein expression; (4) *the use of protease-deficient host strains*: generally, protease expression is linked to cellulase expression in filamentous fungi. The secreted proteases degrade recombinant proteins reducing the yields of expressed proteins. Recombinant cellulase expression in protease-deficient host strains would improve the protein expression yields [33,34].

6.4. Expression in plants

Plants are alternative platforms to microbial and animal cell expression systems for producing recombinant proteins. The most commonly used approaches to express recombinant proteins in plants are (1) expression from the plant nuclear genome; (2) expression from the plastid genome; (3) expression from plant tissues carrying recombinant plant viral sequences. Using these three methods the protein expression titers achieved are generally low. To boost up the recombinant protein expression, some new strategies have been implemented to enhance the production yields. They are (1) co-expression of replicases and transcription factors; (2) the use of strong promoters; (3) increasing the copy numbers of the foreign genes; (4) codon optimization; (5) targeting the recombinant proteins into sub-cellular organelles; (6) protein fusions to improve the stability of the recombinant proteins [35]. The advantages of expressing recombinant proteins in plants are the following: production costs are significantly reduced, the proteins are functionally expressed with glycosylation when compared to *E. coli* (non-glycosylated) or yeast (hyperglycosylated) platforms and expression of cellulase genes itself usually has positive impacts on the plant growth [35].

Cellulases expressed affect the paracrystalline sites of cellulose microfibrils to loosen xyloglucan intercalation promoting the enlargement of the plant cells (NMR spectra). Increased cell size enhanced the rosettes of *Arabidopsis thaliana* [36]. These changes in the plant properties would help in pretreatment of biomass to produce fermentable sugars. Expression of *Acidothermus cellulolyticus* Cel 5A endo-cellulase reduced recalcitrance of the tobacco and maize plants, making biomass softer and easier to be pretreated under mild conditions with high levels of fermentable sugars [37]. *T. fusca* endoglucanases E1, E2 and E5 were successfully transformed and expressed in tobacco, maize and wheat leafs [38]. Details of

Table 2
Heterologous expression of cellulases in microbial hosts.

Source of enzyme gene	Expression host	Enzyme type	Enzyme activity	Reference
Thermonospora YX	*E. coli*	Endoglucanase	5.8 μmol/min/mg × 10^3	[111]
B. subtilis DR	*E. coli*	Endoglucanase	0.82 U/ml	[112]
Xylella fastidiosa	*E. coli*	Endoglucanase	2.39 μKat	[113]
Azoarcus sp. strain BH72	*E. coli*	Exoglucanase	30 U/mg of protein	[114]
Ruminococcus flavefaciens	*E. coli*	Endoglucanase	19.4 μg/min/mg	[115]
Bacillus sp. strain KSM-64	*B. subtilis*	Endoglucanase	21,700 U/L	[116]
Thermomonospora fusca	*S. lividans*	Endoglucanase	10 U/ml	[117]
A. tubingensis	*K. lactis*	Endoglucanase	–	[118]
Cryptococcus sp. S-2	*P. pastoris*	Endoglucanase	4.36 U/mg of protein	[119]
T. reesei	*P. pastoris*	Exoglucanase-7	–	[120]
T. reesei	*P. pastoris*	Exoglucanase-1	–	[121]
Trichoderma reesei QM9414	*S. cerevisiae*	Endoglucanase	–	[122]
Trichoderma reesei QM9414	*S. pombe*	Endoglucanase	–	[122]
T. reesei	*S. pombe*	Exoglucanase-2	Exoglucanase-3.81 U/mg of protein Endoglucanase-0.1 U/mg of protein	[123]
T. reesei	*Y. lipolytica*	Endoglucanase-1	–	[124]
A. fumigatus Z5	*P. pastoris*	β-Glucosidase	101.7 ± 5.2 U/mg of protein	[125]
Candida wickerhamii	*S. cerevisiae*	β-Glucosidase	–	[126]
P. chrysosporium	*P. pastoris*	β-Glucosidase	52 U/mg of protein	[127]

Table 3
Patents on cellulase coding genes and their coding enzymes.

Source of gene	Family	Enzyme type	Enzyme property	Reference
Streptomycetes thermoveolaceous	Streptomycetaceae	Endoglucanase	36 kDa, optimum pH 7 and temperature 60 °C	[128]
Piromyces rhizinjlata	Neocallimastigaceae	Endoglucanase	Optimum pH 6.5 and temperature 40 °C	[129]
C. thermocellum	Clostridiaceae	Exo- and endoglucanase	57 kDa, optimum pH 4.5 and temperature 60 °C	[130]
Bacillus sp.	Bacillaceae	Endoglucanase	Active up to pH 11.5	[131]
Bacillus sp. CBS 670.93	Bacillaceae	Endoglucanase	50 kDa, optimum pH 7 and temperature 60 °C	[132]
Orpinomyces sp.PC-2	Neocallimastiaceae	Exoglucanases A, B and C	Optimum pH between 5 and 6 and temperature between 40 and 60 °C	[133]
Bacillus sp.	Bacillaceae	Endoglucanase	Active up to pH 11.5	[134]
Bacillus sp. NCIMB 40250	Bacillaceae	Endoglucanase 1–4	Endo 1: 57 kDa, pI of the protein is 4, stable up to pH 9–10	[135]
Rhodothermus marinus	Rhodothermaceae	Exoglucanase 12A	Family 12 GH, optimal pH 4 to 8 and optimum temperature of 90 °C	[136]
Thermotoga maritima	Thermotogaceae	Endoglucanase	35 kDa	[137]
Acidothermus cellulolyticus	Acidothermaceae	Endoglucanase	42 kDa, optimal pH 5 and temperature 65 °C	[138]
Aspergillus niger 400	Trichocomaceae	Endoglucanase	Optimum pH 3.5 and temperature 40 °C	[139]
Caldicellulosiruptor sp.	Thermoanaerobacterales Family III	Endoglucanase B	46.7 kDa, optimum pH 7 and temperature 50 °C.	[140]
T. reesei	Hypocreaceae	β-Glucosidase	–	[141]
T. longibrachiatum	Hypocreaceae	Endoglucanase I–III	Endo-glucanase I: ~ 47-49 kDa, pI 4.7 and optimim pH 5 Endo-glucanase II: ~ 35 kDa, pI 5.5 and optimum pH 5 Endo-glucanase III: ~ 22-27 kDa, pI 7.4 and optimum pH 5.5-6.0	[142]

various heterologously expressed microbial cellulases are listed in Table 2.

7. Engineering of filamentous fungi

7.1. Rational design for enhancing cellulase expression

Filamentous fungi natively secrete various cellulases when growing on lignocellulose wastes. Improving cellulase expression by random mutagenesis is the classical approach. Detailed analysis of secretome, complete genome information and whole cell transcriptome analysis by microarrays showed that multiple transcription factors systematically regulate expression of cellulases [39,40]. It has been recognized that some fungal species have independently developed different mechanisms in the induction of cellulase genes in response to various inducers. Rational engineering of the microbial regulatory network is the most advanced approach and is considered to be able to further improve productivity and specificity of cellulase secretion.

Cellulase expression and secretion can be induced in *T. reesei* (*Hypocrea jecorina*) by small molecular metabolites such as cellobiose, lactose, sophorose and xylose. Several positive (XYR1, ACE2, and HAP2/3/5) and negative (ACE1 and CRE1) regulators are involved in the regulation of cellulase gene expression. These regulators are zinc finger proteins, binding to cellulase gene promoters to regulate cellulase expression [41]. *ace1* knock-out strain showed increased expression of cellulases and hemicellulases, speculating that the ACEI protein acts as a repressor for cellulase and hemicellulase gene expression [42]. *ace2* knock-out strain reduced the transcription levels of cellulase mRNAs by 30% and cellulase activity by 70% [43]. Deletion of *xyr1* gene eliminated cellulase induction both on cellulose and sophorose [44]. Effect of constitutive over-expression of XYR1 gene on the production of cellulase enzymes needs to be studied.

Similarly, the transcriptional profile and knock-out studies of various transcription factors in *Neurospora crassa* identified two

transcription factors *clr-1* and *clr-2* being required for induction and expression of cellulases. *clr-1* knock-out strain poorly grew on cellobiose suggesting the importance of this protein in cellobiose sensing mechanism. During carbon catabolite repression, basal level expression of CLR-1 and cellulase genes produces proteins at low levels. The expressed cellulases are secreted outside producing cellobiose, which enters the cell and activates CLR-1. The activated CLR-1 further activates cellodextrin transporters and β-glucosidase genes. The activated CLR-1 is necessary to increase the clr-2 gene expression and self-regulate its own expression. CLR-2 induces cellulase and hemicellulase gene expression in a heterocomplex along with CLR-1 [45]. Creating fungal strains that over-express positive gene regulators with negative regulator genes being knocked out would improve the cellulase expression.

7.2. Random mutagenesis for enhanced cellulase expression

The complete depolymerization of cellulose polymer needs the synergistic action of three principle cellulases. The use of whole cell cultures is a good option. It confers multiple advantages such as decrease in the enzyme production cost, possibility for expressing complete spectrum of enzymes and relatively simple scale up process for bulk and fine chemical production [46]. Therefore, a whole cell engineering of filamentous fungi for elevated cellulase expression is an ideal choice.

T. reesei KCTC 6950 was mutated by proton beam irradiation to improve overall cellulase expression and optimize the ratio of three principle cellulases. Colony level screening of 1000 mutants gave five mutants (MT 1–5) for their ability to overexpress cellulases by using the plate screening methodology. From the selected five mutants, strain MT-2 showed 165% increase in filter paper (Fpase)/exoglucanase activity, 146% increase in carboxy methyl cellulase (CMCase)/endoglucanase activity and 313% increase in β-glucosidase activities compared to the wild type strain in submerged fermentation. Improved expression was confirmed by SDS-PAGE and MUG (4-methylumbelliferyl-β-D-glucoside)-zymogram assay. The random mutations did not affect any biochemical properties of these enzymes [47].

Spores of *Aspergillus* sp. SU14 were mutated sequentially and repeatedly using Co^{60} γ-rays, UV irradiation and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG). Out of 500 mutants a mutant strain *Aspergillus* sp. SU14-M15 showed 2.2-fold improved cellulase expression than wild type strain. Further optimizing the conditions of solid-state fermentation using basal wheat bran medium gave 8.5-fold cellulase yield compared to the wild type strain [48].

Thermophilic fungus *H. insolens* TAS-13 was exposed to five different mutagens in parallel, UV irradiation, NTG, nitrous acid (HNO_2), ethyl methyl sulfonate (EMS) and ethidium bromide (EtBr). After first round of mutagenesis, 26 mutants were isolated and among them five best mutants (TASUV-4, TASNTG-7, TASHN-4, TASEB-2 and TASEMS-1) were selected for their increased cellulase expression ability. These selected mutants from the first round of mutagenesis were further mutated in parallel with all five mutagens. Totally 33s generation mutants were selected. Among them a mutant strain TAS-13UV-4NTG-5 was found to be the best one producing 43.2% more of CMCase, 60.2% more of FPase and 59.8% more of β-glucosidase activity than the first generation mutant TAS-13UV-4. This mutant was genetically stable up to many generations possessing the high cellulase expressing ability [49].

Along with the random mutagenesis of the genomic DNA, site specific mutagenesis of the ribosomal DNA that generates engineered ribosome for enhanced cellulase expression is worth investigating as it improved hemicellulase expression in the *Streptomyces viridochromogenes* M11 [50].

8. Genetic engineering of cellulases

Engineering the cellulase enzymes to improve their properties to meet the robust industrial applications is often required. Random mutagenesis, site specific mutagenesis or their combination have been used to get tailor made enzymes for industrial applications [51]. Few recent examples on engineering cellulases are described here.

Detergents used in cloth washing have a pH of 10. Therefore, cellulases to be used in detergents should show maximal activity and stability at pH 10. Molecular modeling of Egl-237 (endoglucanase from *Bacillus* sp. KSMS-237) was constructed using homology modeling taking crystal structure of CelK alkaline cellulase (*Bacillus* sp. KSM635). Based on the data obtained, six amino acids from 357 to 362 (Gly–Lys Ser–Asn–Ala–Thr) in the loop region were replaced with three amino acid short peptide chains. Three different peptide chains with the compositions Ala–Gly–Ala, Ala–His–Ala and Ala–Arg–Ala were used to generate enzyme variants. Loop shortened cellulase variants were expressed in *Bacillus subtilis*, purified and tested for improved properties. The cellulase variant with Ala–Gly–Ala showed maximal activity at pH 10 which is 1 pH unit higher than the wild type cellulase (pH 9). The remaining two cellulase variants were active at pH 9.6 and 95% improvement in specific activity [52].

Thielavia terrestris cellulase was engineered by homology modeling taking crystal structure of *H. insolens* endoglucanase V (EGV). Based on the results from modeling a combination of single and multiple mutations were created to generate multiple cellulase variants with improved properties. Replacement of Glu residue with His at 119 position increased specific activity of this enzyme from 35% to 92% (*H. insolens* specific activity was set as 100%) and the relative activity at pH 10 was increased from 27% to 62% when compared to wild type. Replacement of two cysteine residues at positions 16 and 86 with Met and Gly eliminated disulfide bonds reducing the temperature stability from 81 °C to 59.2 °C and improved specific activity from 100% to 103% (*H. insolens* specific activity was set as 100%) [53].

Inactivation of cellulases by lignin is one of the major bottlenecks in enzymatic hydrolysis of biomass. *T. reesei* cel6A's resistance to lignin inactivation was improved by 15% by replacing six amino acids (Lys-129 to Glu-129, Ser-186 to Thr-186, Ala-322 to Asp-322, Gln-363 to Glu-363, Ser-413 to Pro-413, and Arg-410 Gln-410) [54].

Inhibition of cellulases at higher glucose concentrations is one of the bottlenecks to be overcome for efficient enzymatic hydrolysis of cellulose. Based on homology modeling of family six cellulases from *P. chrysosporium,* the *T. reesei* cel6A's was engineered. Replacement of selected amino acids decreased enzyme inhibition to higher glucose concentrations by 1.43- and 3.22-fold when compared to wild type (Tyr-98 to Lys or Leu, Leu-131 to Ile or Val, Ser-182 to Lys or Tyr, Gly-359 to Gln, Arg-404 to Gln) and *H. insolens* cel6A (Tyr-107 to Lys, Tyr-107 to Leu, Gln-139 to Thr, Leu-141 to Val) [55].

Endoglucanase 5 from *Humicola insolens* was engineered to resist surfactants at alkaline pHs. Two single (Ala-162-Pro; Lys-166-Glu) and one double mutant (Ala-162-Pro Lys-166-Glu) were generated. These protein variants on average showed 3.7 times higher activities when compared to the original cellulase [56].

T. reesei Cel6A was engineered (Ser-413-Pro). A single amino acid change increased enzyme optimal temperature (+5.6 °C), alkalophilicity (+1.25 pH units), thermostability (11.25 times) and T_{50} of its β-glucan hydrolyzing activity (+5 °C) when compared to the original cellulase [57].

A newly discovered cellulase 11AG8 from *Actinomycete* sps was identified as an endoglucanase. It is 386 amino acids long with a catalytic core and a CBM. A truncated version comprising only the catalytic core (221 amino acids) with cellulase function was

functionally expressed in *Streptomyces* for applications in textile industry [58].

A heterologous gene construct comprising *T. reesei cbh1* catalytic core and its linker fused with *A. cellulolyticus* endoglucanase catalytic core was functionally expressed in *T. reesei*. The fusion protein showed superior activity within 6 h bringing 20% cellulose conversion [59].

A new EGIII like cellulase was recognized in *Humicola grisea var. thermoidia*. It showed a higher thermostability when compared to its homolog EGIII from *T. reesei*. Based on the amino acid sequence comparison, changes were made to replace some the amino acids to improve the stability of EGIII from *T. reesei*. Two enzyme variants with point mutations Gly-170-Cys and Val-210-Cys improved stability 1.03 and 1.29 times, respectively, than the wild type. Improved stability of these enzyme variants is ascribed to the introduction of new disulfide bonds. Surprisingly, same mutations significantly reduced the specific activity of these enzymes in comparison with wild type [60].

9. Activity assay of cellulases

Activity assays for detecting the cellulase enzymes have been conducted using both natural and synthetic substrates. There are overall activity assay for measuring total cellulase activity and also individual assays for each principle cellulase [61,62]. Many activity assay methods have been reported, but here we describe only those routinely used in biomass conversion.

1. *FPA total cellulase activity* (*FPase activity*): It is the key assay method developed by Mandels et al. to determine the total cellulase activity. In this method 1 × 6 cm strip of Whatman no. 1 filter paper is used as the standard substrate. Total amount of glucose obtained due to cellulase activity is measured using different reducing sugar detection methods such as 3,5-dinitrosalicylic acid (DNS), glucose oxidase (GOD) or high performance liquid chromatography (HPLC) [61].
2. *Endoglucanase activity* (*carboxy methyl cellulase activity*): The endoglucanase activity is measured using cellulose derivatives with a high degree of polymerization such as carboxy methyl cellulose (CMC). The total amount of reducing sugars obtained is determined using DNS, GOD, HPLC or Somogyi–Nelson methods. Alternatively, reduction in the viscosity of the CMC due to enzyme activity can also be measured using a viscometer [61,62].
3. *Exoglucanase activity* (*Avicelase activity*): The exoglucanase activity is measured on crystalline cellulose Avicel as a substrate. The total amount of reducing sugars obtained is measured using DNS, GOD or HPLC methods [61,62].
4. β-*Glucosidase activity*: The glucosidase activity is measured using cellobiose as the substrate. The amount of glucose released is analyzed by HPLC [61,62].
5. Alternatively, detection of cellulase enzyme activities is also possible using synthetic substrates. These substrates undergo color changes during the course of hydrolysis. The change in color of the reaction system is the basis for measurement of enzyme activities. RBB (Remazol brilliant blue)-dyed CMC and Avicel can be used as substrates for endo- and exoglucanase activities, respectively. The release of RBB dye from the substrate can be detected at 595 nm. Similarly, 4-nitrophenyl glucoside is used as the substrate for the β-glucosidase activity. The released 4-nitrophenol can be detected at 410 nm. Similarly, the fluorescent-labeled substrate (4-methylumbelliferyl glycoside) can also be used. The released 4-methylumbelliferone is measured at 354 nm [61,63,64].
6. Automated measurement of cellulase activity using different modern techniques is also possible. These techniques are extensions of classical assays described above. Modern techniques include quartz crystalline microbalance, miniaturized colorimetric assay, automated FPA, fluorescent microfibrils and amperometric cellobiose dehydrogenase biosensor. Advantages of using these modern methods include user friendliness, suitability for use in high throughput screening, more reliable results and reduced use of reagents thus more economic choice [61].

10. Recovery and reuse of cellulases

One of the major bottlenecks that hinder the commercialization of biomass conversion is the high cost of cellulases used in the hydrolytic processes. It has been estimated that the cost of enzymes accounts for up to 50% of the hydrolytic processes [65,66]. Cellulases are found to be very stable in the hydrolytic process, existing either in the solid phase bound to residual substrates or present in the supernatant liquid phase. Recovery and reuse of enzymes from both phases would reduce the enzyme cost favoring commercialization of the biochemical processes. Therefore, considerable attention has been focused on recycling and reuse of cellulases [67].

Cellulases from *T. reesei* were recovered and reused by simple adsorption and desorption over weakly acidic ion exchange resin WK10. Optimal pH values for adsorption and desorption were 4 and 8, respectively, and the optimal adsorption and desorption times were both 5 h. Almost 100% of the initial cellulase activity was recovered under the optimal conditions with the supplement of β-glucosidase, which was unable to be efficiently recovered due to its strong adsorption (95.7%) but poor desorption (1.9%) [68].

Douglas fir plant pulp prepared by ethanol pretreatment (EPMS) was mixed with Celluclast (FPU) (filter paper unit) and external β-glucosidase (IU) in a ratio of 1:2. After hydrolysis solid phase cellulase fraction I was recovered from residual EPMS. Fractions II and III were recovered from the supernatant by re-adsorption over fresh substrate and by ultra-filtration. Cellulases were shown to retain most of their activity and 51% of the applied cellulases were recovered from the EPMS. By using Langmuir adsorption isotherm 82% of free cellulases in supernatant could be recovered by the addition of fresh substrates [67].

Adsorption profiles of *T. reesei* cellulases over different cellulose substrates i.e. Avicel, xylan and potato pulp waste were tested. Adsorption reached maximum within 10 min at 30 °C. Enzymes showed high affinity for potato pulp. After 24 h of hydrolysis at 50 °C, more than 55% of enzyme activity remained in the supernatant indicating the possibility for recovery and reuse of these enzymes. Enzyme recovery was 55% on addition of fresh pulp achieving 32% saccharification on further incubation [69].

T. reesei β-glucosidase was adsorbed over Kieselgur granules at neutral pH. Enzyme bound carrier granules were mixed into the hydrolytic reaction containing Avicel as a substrate at pH 3.5. Acidic pH of the reaction releases the enzyme from the carrier molecules bringing hydrolysis of the Avicel releasing glucose. After enzyme reaction solid phase cellulase present on the residual Avicel was separated by centrifugation. The soluble enzyme present in the supernatant was re-adsorbed back onto the carrier granules by neutralizing the reaction to pH7.0. By this approach 50% of the initial enzyme was recovered by re-adsorption [70].

11. Industrial applications of cellulases

Cellulases have a wide spectrum of applications in various industries. Traditionally, they are applied in food and brewery production, animal feed processing, detergent production and laundry, textile processing and paper pulp manufacture. Due to the crisis in sustainable supply of fossil fuel and the increased

demand for production of biofuels and chemicals from renewable resources, their applications in cellulose biorefinery for producing fermentable sugars are expected to rapidly increase in the foreseeable future. Recent publications and patents describing the applications of cellulases in various industries are briefly summarized below [3,4].

11.1. In textile industry

Cellulases are routinely used in textile processing and finishing of the cellulose based textiles. Cellulases are effective in removing hairiness of the cellulose threads which are used in textile production with negligible weight loss, developing smooth and glossy appearance of the cloth and imparting color brightness. Enzymatic washing of cotton cloths generally involves acidic cellulase from *T. reesei* and neutral cellulases from *H. insolens* [3,71]:

1. Truncated *Thermonospora fusca* endoglucanase E5 was tested for its abrasion performance on denim jeans in stonewashing applications. Applying 13,500 RBB-CMC units of enzyme for washing at 60 °C for 60 and 90 min gave excellent abrasion levels of 5.5 and 7, respectively. Combined desize and bleach processes did not have any negative impact on the abrasion efficiency of this enzyme [72].
2. *Chrysosporium lucknowense* strain C-1 isolated from the alkaline soil is capable of producing multiple cellulases which are active at neutral and alkaline pHs. A composition of these cellulases were tested for their washing performance on denim jeans in stone wash applications. These enzyme preparations showed good abrasion/color reduction performance (+4 to +6) and lowest levels of backstaining (2) which are ideal for textile washing applications and superior in comparison with commercial enzyme preparations [73].
3. A high level CBH II expressing *T. reesei* strain ALK03798 was constructed, cellulases produced from this strain contain higher concentrations of CBH II. This enzyme preparation was used in bio-finishing of cotton textiles. After washing, cotton cloth had improved properties such as high weight loss (5%) due to removal of pill and fuzz after 200 cycles of abrasion, imparting good visual appearance without losing tensile strength of the cloth when compared to cellulase preparation that had all cellulases [74].

11.2. In detergent industries

Addition of enzyme cocktail containing cellulases, lipases and proteases in the detergent compositions is a new trend followed by many detergent industries in recent years [3]. Alkaline cellulases present in the detergent composition can pass through the interfibril spaces easily and help in effective removal of stains from cloths. Additionally, cellulases process the cellulose fibrils impart color brightness and smoothness to the cloths even after repeated washing [3,4]:

1. Cellulases from the *Bacillus* sps 669.63 and 670.93 were mixed in the concentrations of 0.01–0.2 mg/ml, giving the ratio of tensile strength loss to antipilling properties below 0.2–0.02. At this ratio, detergents have excellent properties of antigreying effect (5, Δ REM), softening of fabrics (rating 2.2–2.3), least fiber damage (0.025 mU), inhibiting color deterioration and inhibiting wrinkles [75].
2. A detergent composition containing *Trichoderma longibrachiatum* exo-cellobiohydrolase I and endoglucanase were added at the ratios of 10:1–400:1. At these ratios detergents had superior cleaning capacity (91% reflectance), imparting high softness to the cloths and reduced tensile strength [76].
3. Different compositions of granular detergent were made comprising truncated *Trichoderma sps* cellulases within the range of 0.0001–0.05%. These detergents with enzymes were superior in cleansing properties in comparison with that without the enzymes [77].

11.3. In food processing industries

Cellulases have broader applications in food processing industry. Cellulases in combination with other enzymes (xylanases and pectinases), collectively known as macerating enzymes, have been used in extraction and clarification of fruit and vegetable juices to increase their yields. Macerating enzymes reduce viscosity; improve cloud stability and aromatic properties of the fruit juices and their pulps during processing. The enzyme pretreatment reduces the risk of membrane fowling during juice filtration [78]:

1. Carboxy methyl cellulase was added to the dough to improve bread quality. Addition of 250 IU of enzyme for every100 g of flour improved the maximal volume ($635 \pm 25.30\ cm^3$), specific volume ($3.99 \pm 0.14\ cm^3/g$) and farinographic parameters (water absorption, arrival time, dough development time and dough stability time) of the bread [79].
2. Enzyme mixture containing cellobiohydrolase ($29\ U\ ml^{-1}$) and pectin lyase ($50\ U\ ml^{-1}$) from *A. niger* was added to the apple homogenate and incubated at 40 °C and 150 rpm for 24 h. After enzyme hydrolysis, a cloudy apple juice was obtained which was stable for several months [80].
3. Palm date (*Phoenix dactylifera*) is a good source of sugars as it is enriched with carbohydrates (up to 80%). In recent days sugar syrup obtained from this fruit is the major ingredient in confectionary and food industries. Addition of 1% enzyme preparation containing cellulase and pectinase increased sugar yield from this fruit by 22.3% reducing the moisture content from 18.8% to 15.5% with a significant improvement in the mineral composition of the syrup [81].
4. Cellulases were used along with xylanases in the production of crispy bread and biscuits. Addition of carboxy methyl cellulase ($9300\ U\ g^{-1}$), exoglucanase ($380\ U\ g^{-1}$) and xylanase ($25{,}000\ U\ g^{-1}$) for every kilogram of flour improved the dough properties and increased the process capacity by decreasing the need for liquids used for dough preparation [82].

11.4. In animal feed industries

Cellulases along with hemicellulases are used in the production of animal and poultry feeds. Addition of enzymes during feed processing brings partial hydrolysis of cellulase and hemicellulase components present in the silage, dehulling of cereal grains and better emulsification of feeds. Partially digested feed after entering into animals' digestive track would be digested more completely providing more nutrients, hence better growth of animals [83,84]:

1. Commercial cellulase composition cytolase123 (Genecor International, USA) was separated into four fractions A, B, C and D using anion exchange chromatography. Each fraction has its own variable cellulase and hemicellulase enzyme compositions confirmed by different enzyme activity assays. Fraction A acts on the carbohydrate polymer generating free sugar ends, which are further hydrolyzed by rumen bacteria producing fermentable sugars with small increase in effluent flow. Fractions B and C alone or in combination supply adequate fermentable

sugars without increasing effluent flow. Fraction D alone has adverse affect on depolymerizing complex carbohydrates and increasing effluent flow. Effects of different enzyme fractions on rye grass were tested to detect any improvements on the silage properties. Fraction A alone or in combination with B or C, alternatively, fractions B and C together, improved the fiber structure and chemical compositions of rye grass silage [85].
2. Cellulase (*T. reesei*) and glucose oxidase (*A. niger*) were applied at a rate of 350,000 IU (for both activities) per ton of forage. Addition of these enzymes improved the aerobic stability of the forage even after 30 days of ensiling by reducing the mold and yeast growth. A combination of *Lactobacillus buchneri* to this enzyme cocktail synergistically enhanced aerobic stability of silage [86].

11.5. In removal of bacterial biofilm

When binding to an inert surface for a longer time, bacterial cells secrete sticky extracellular polysaccharides (EPS). The EPS develops a matrix for the growth and division of the existing bacterial cells forming a growing biofilm. The EPS seems to resemble the plant cell wall polysaccharides. Several carbohydrate degrading enzymes and proteases have been used to get rid of this bacterial biofilm [87].

1. Slime layers are formed routinely on the walls of cooling towers in waste water treatment and paper industries. On using an enzyme combination consisting of polysaccharide degrading enzymes in a ratio of two parts of cellulase, one part of α-amylase and one part of protease were used together in 2–100 ppm (ppm) for 31 days at pH 9 prevented slime layer formation significantly for months. It is postulated that α-amylase and protease together created nicks in the slime layer providing the access sites to cellulase to digest exopolymer more effectively [88].
2. An enzyme combination for targeting exopolysaccharide layer was developed. It included α-glucosidase (Sigma Chemical Co), galactosidase, galacturonidase, rhamnosidase, xylosidase, fucosidase and arabinosidases (Novozymes, Novo Nordisk and Miles). The enzyme composition was tested over slime layers formed by *Klebsiella pneumoniae*, IPC 500 (Institute of Paper Chemistry) and *Pseudomonas aeruginosa* ATTC 10145 in 20–200 ppm for 72 h and 144 h, respectively. The enzyme treatment for 72 h inhibited bacterial attachment thereby slowing down the slime layer formation [89].
3. Kits containing different enzyme compositions were developed for slime mold removal. The kits included different proportions of (1–6 wt%) cellulases, hemicellulases, proteases, amylases and esterases. They were tested for their ability to remove *P. aeruginosa* biofilm on solid surfaces. At acidic, basic and neutral pHs, the kits were able to remove 70–80% of slime mold [90,91].

11.6. In biorefinery

Production of value-added chemicals and biofuels from renewable biomass feedstocks is nowadays a hot topic worldwide. Depolymerization of plant biomass to produce fermentable sugars is one of the prerequisites. Biological pretreatment of biomass is environmental friendly and harbors multiple advantages [92]:

1. Enzyme cocktail containing 3.5 FPU g^{-1} (Celluclast and Novozymes 188 in 5:1 ratio) and amylase 1.75 g kg^{-1} dry mass (NS50033, Novozymes) was mixed with corn silage at 5% final dry matter content. Silage was incubated at 250 rpm, 50 °C for 6 h yielding 72% of fermentable sugars. Inclusion of heat pretreatment step at 190 °C for 12 min followed by enzyme treatment increased the yields of fermentable sugars to 83%. Hydrolysate generated could be used to produce bio-ethanol or other value-added chemicals from lignocellulose [93].
2. *S. cerevisiae* strain displaying *T. reesei* EG II, CBH II and *Aspergillus aculeatus* β-glucosidase were developed. Using this strain bio-ethanol was produced from amorphous cellulose and ionic liquid-swollen cellulose separately by fermentation. Yields of ethanol reached 2.1 g l^{-1}after 60 h [94,95].
3. *T. reesei* F-418 was cultivated on alkali pretreated rice straw by solid state fermentation yielding cellulase concentrations of 16.2 IU g^{-1}after 5 days of incubation at pH4.8. Saccharification of alkali pretreated rice straw was conducted taking 1.2 IU $ml^{-1}$of cellulase enzymes in a final volume of 5% (w/v). After 16 h of incubation glucose concentration levels reached 1.07% [96].
4. *Penicillium janthinellum* mutant EU1was grown in 10% bagasse secreting 10 FPU g/l of cellulase bringing 72% hydrolysis releasing glucose and cellobiose. Simultaneous saccharification and fermentation with *Lactobacillus lactis* (RM2-24) yielded 73 g l^{-1} of lactic acid from 100 g l^{-1} of α-cellulose with a yield of 0.73 g per g of cellulose at a productivity of 1.52 g/l/h [97].

12. Conclusion

Application of biotechnological process to produce value-added chemicals such as ethanol and lactic acid from renewable lignocellulose biomass resources has received much attention in recent decade. The key step is the depolymerization of biomass polymers into fermentable sugars. Depolymerization of lignocelluloses is generally done by either chemical route using mineral acids or biological route using enzymes and whole microbial cells. Acid catalysis requires harsh conditions along with the formation of sugar degradation products such as furfural and 5-hydroxymethylfurfural which inhibit microbial growth in the successive fermentation steps. Enzymatic depolymerization does not generate sugar degradation products as the reactions are usually conducted under mild conditions. This avoids additional detoxification steps before successive microbial fermentation. Genetic engineering of the existing cellulases with high specific activity, resistance to feedback inhibition and stability to robust conditions is a practical option. The production of recombinant proteins in heterologous hosts is generally limited to mg or g levels per liter. Strategies such as codon optimization, screening multicopy strains with high expression levels, co-expression of helper proteins and cell organelle engineering are worth considering to improve the enzyme production level. Alternatively, as the complete depolymerization of cellulose needs the synergistic action of a spectrum of cellulases, whole cell genome engineering to improve the production of all cellulases is a better choice. Tailor-made enzymes, cost-effective large scale production, recycle and reuse of cellulases and robust cellulase modeling for industrial applications would collectively improve the high sugar yield in enzymatic hydrolysis of cellulose for commercial applications.

Acknowledgments

This research is supported by the Science and Engineering Research Council (SERC) of the Agency for Science, Technology and Research (A*STAR) of Singapore (SERC Grant nos. 0921590132 and 1124004027).

References

[1] Heredia A, Jiménez A, Guillén R. Composition of plant cell walls. Z Lebensm Unters Forch 1995;200:24–31.
[2] Juturu V, Wu JC. Microbial xylanases. Engineering, production and applications. Biotechnol Adv 2012;30:1219–27.
[3] Kuhad RC, Gupta R, Singh A. Microbial cellulases and their industrial applications. Enzyme Res 2011. http://dx.doi.org/10.4061/2011/280696.
[4] Karmakar M, Ray RR. Current trends in research and application of microbial cellulases. Res J Microbiol 2011;6:41–53.
[5] Medie FM, Davies GJ, Drancourt M, Henrissat B. Genome analyses highlight the different biological roles of cellulases. Nat Rev Microbiol 2012;10:227–34.
[6] Saranraj P, Stella D, Reetha D. Microbial cellulases and its applications: a review. I J Biochem Biotech Sci 2012;1:1–12.
[7] Doi RH, Kosugi A. Cellulosomes. plant-cell-wall-degrading enzyme complexes. Nat Rev Microbiol 2004;2:541–51.
[8] Medie FM, Davies GJ, Drancourt M, Henrissat B. Genome analyses highlight the different biological roles of cellulases. Nat Rev Microbiol 2012;10:227–34.
[9] Desvaux M. *Clostridium cellulolyticum*. model organism of mesophilic cellulolytic clostridia. FEMS Microbiol Rev 2005;29:741–64.
[10] Bayer EA, Chanzy H, Lamed R, Shoham Y. Cellulose, cellulases and cellulosomes. Curr Opin Struct Biol 1998;8:548–57.
[11] Lo Leggio L, Larsen S. The 1.62 Å structure of *Thermoascus aurantiacus* endoglucanase: completing the structural picture of subfamilies in glycoside hydrolase family 5. FEBS Lett 2002;523:103–8.
[12] Muñoz IG, Ubhayasekera W, Henriksson H, Szabó I, Pettersson G, Johansson G, et al. Family 7 cellobiohydrolases from *Phanerochaete chrysosporium*: crystal structure of the catalytic module of Cel7D (CBH58) at 1.32 Å resolution and homology models of the isozymes. J Mol Biol 2001;314:1097–111.
[13] Sanz-Aparicio J, Hermoso JA, Martínez-Ripoll M, Lequerica JL, Polaina J. Crystal structure of β-glucosidase A from *Bacillus polymyxa*: insights into the catalytic activity in family 1 glycosyl hydrolases. J Mol Biol 1998;275:491–502.
[14] Violot S, Aghajari N, Czjzek M, Feller G, Sonan GK, Gouet P, et al. Structure of a full length psychrophilic cellulase from *Pseudoalteromonas haloplanktis* revealed by x-ray diffraction and small angle x-ray scattering. J Mol Biol 2005;348:1211–24.
[15] Kasana RC, Gulati A. Cellulases from psychrophilic microorganisms: a review. J Basic Microbiol 2011;51:572–9.
[16] Ubhayasekera W, Muñoz IG, Vasella A, Ståhlberg J, Mowbray SL. Structures of *Phanerochaete chrysosporium* Cel7D in complex with product and inhibitors. FEBS J 2005;272:1952–64.
[17] Taylor TJ, Vaisman II. Discrimination of thermophilic and mesophilic proteins. BMC Struct Biol 2010;10(1):S5, http://dx.doi.org/10.1186/1472-6807-10-S1-S5.
[18] Li Duo-Chuan, Li An-Na, Papageorgiou Anastassios C. Cellulases from thermophilic fungi: recent insights and biotechnological potential. Enzyme Res 2011. http://dx.doi.org/10.4061/2011/308730.
[19] Willick GE, Seligy VL. Multiplicity in cellulases of *Schizophyllum commune*. Eur J Biochem 1985;151:89–96.
[20] Kubicek C. The cellulase proteins of *Trichoderma reesei*: structure, multiplicity, mode of action and regulation of formation enzymes and products from bacteria fungi and plant cells. In: Scheper T, Belkin S, Doran PM, Endo I, Gu MB, Hu WS, Nielsen J, Stephanopoulos GN, Ulber R, Zeng AP, Zhong JJ, Zhou W, editors. Advances in biochemical engineering/biotechnology. Germany: E-Publishing Inc.; 1992. p. 1–27.
[21] Haab D, Hagspiel K, Szakmary K, Kubicek CP. Formation of the extracellular proteases from *Trichoderma reesei* QM 9414 involved in cellulase degradation. J Biotechnol 1990;16:187–98.
[22] Zhou F, Olman V, Xu Y. Large-scale analyses of glycosylation in cellulases. Genomics Proteom Bioinform 2009;7:194–9.
[23] Langsford ML, Gilkes NR, Singh B, Moser B, Miller Jr RC, Warren RA, et al. Glycosylation of bacterial cellulases prevents proteolytic cleavage between functional domains. FEBS Lett 1987;25:163–7.
[24] Hayashida S, Yoshioka H. The role of carbohydrate moiety on thermostability of cellulases from *Humicola insolens* YH-8. Agric Biol Chem 1980;44:481–7.
[25] Bansal P, Hall M, Realff MJ, Lee JH, Bommarius AS. Modeling cellulase kinetics on lignocellulosic substrates. Biotechnol Adv 2009;27:833–48.
[26] Linger JG, Adney WS, Darzins A. Heterologous expression and extracellular secretion of cellulolytic enzymes by *Zymomonas mobilis*. Appl Environ Microbiol 2010;76:6360–9.
[27] Delgado OD, Martínez MA, Abate CM, Siñeriz F. Chromosomal integration and expression of green fluorescent protein in *Zymomonas mobilis*. Biotechnol Lett 2002;24:1285–90.
[28] Lejeune A, Eveleigh DE, Colson C. Expression of an endoglucanase gene of *Pseudomonas fluorescens* var. cellulosa in *Zymomonas mobilis*. FEMS Microbiol Lett 1988;49:363–6.
[29] Dees CH. Genetically enhanced cellulase production in *Pseudomonas cellulosa* using recombinant DNA technology. US Patent 5958740; 1999.
[30] Binnie C, Douglas Cossar J, Stewart DIH. Heterologous biopharmaceutical protein expression in *Streptomyces*. Trend Biotechnol 1997;15:315–20.
[31] Herai S, Hashimoto Y, Higashibata H, Maseda H, Ikeda H, Ōmura S, et al. Hyper-inducible expression system for *streptomycetes*. Proc Natl Acad Sci USA 2004;101:14031–5.
[32] Anné J, Maldonado B, Van Impe J, Van Mellaert L, Bernaerts K. Recombinant protein production and streptomycetes. J Biotechnol 2012;158:159–67.
[33] Ward OP. Production of recombinant proteins by filamentous fungi. Biotechnol Adv 2012;30:1119–39.
[34] Sharma R, Katoch M, Srivastava P, Qazi G. Approaches for refining heterologous protein production in filamentous fungi. World J Microbiol Biotechnol 2009;25:2083–94.
[35] Streatfield SJ. Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 2007;5:2–15.
[36] Park YW, Tominaga R, Sugiyama J, Furuta Y, Tanimoto E, Samejima M, et al. Enhancement of growth by expression of poplar cellulase in *Arabidopsis thaliana*. Plant J 2003;33:1099–106.
[37] Brunecky R, Selig M, Vinzant T, Himmel M, Lee D, Blaylock M, et al. In planta expression of *A. cellulolyticus* Cel5A endocellulase reduces cell wall recalcitrance in tobacco and maize. Biotechnol Biofuels 2011;4:1, http://dx.doi.org/10.1186/1754-6834-4-1.
[38] Lebel EG,, Heifetz, Peter B.Ward, Eric R, Uknes, Scott J. Transgenic plants expressing a cellulase. US patent 7361806; 2008.
[39] Stricker A, Mach R, de Graaff L. Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*). Appl Microbiol Biotechnol 2008;78:211–20.
[40] Jun H, Kieselbach T, Jönsson LJ. Enzyme production by filamentous fungi: analysis of the secretome of *Trichoderma reesei* grown on unconventional carbon source. Microb Cell Fact 2011;10:68, http://dx.doi.org/10.1186/1475-2859-10-68.
[41] Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B. Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol Biofuels 2009;2:19.
[42] Aro N, Ilmén M, Saloheimo A, Penttilä M. ACEI of *Trichoderma reesei* is a repressor of cellulase and xylanase expression. Appl Environ Microbiol 2003;69:56–65.
[43] Stricker AR, Trefflinger P, Aro N, Penttilä M, Mach RL. Role of Ace2 (activator of cellulases 2) within the xyn2 transcriptosome of *Hypocrea jecorina*. Fungal Genet Biol 2008;45:436–45.
[44] Stricker AR, Grosstessner-Hain K, Würleitner E, Mach RL. Xyr1 (xylanase regulator 1) regulates both the hydrolytic enzyme system and D-xylose metabolism in *Hypocrea jecorina*. Eukaryot Cell 2006;5:2128–37.
[45] Coradetti ST, Craig JP, Xiong Y, Shock T, Tian C, Glass NL. Conserved and essential transcription factors for cellulase gene expression in ascomycete fungi. Proc Natl Acad Sci USA 2012;109:7397–402.
[46] Fukuda H, Kondo A, Tamalampudi S. Bioenergy. Sustainable fuels from biomass by yeast and fungal whole-cell biocatalysts. Biochem Eng J 2009;44:2–12.
[47] Jung YR, Shin HY, Yoo HY, Um Y, Kim SW. Production of cellulases and high-levels of β-glucosidase in *Trichoderma reesei* mutated by proton beam irradiation. Korean J Chem Eng 2012;29:925–30.
[48] Vu VH, Pham TA, Kim K. Improvement of fungal cellulase production by mutation and optimization of solid state fermentation. Mycobiology 2011;39:20–5.
[49] Javed MM, Haq IU, Mariyam I. Multistep mutagenesis for the over-expression of cellulase in *Humicola insolens*. Pak J Bot 2011;43:6679.
[50] Liu Z, Zhao X, Bai F. Production of xylanase by an alkaline-tolerant marine-derived *Streptomyces viridochromogenes* strain and improvement by ribosome engineering. Appl Microbiol Biotechnol 2013;10:4361–8.
[51] Dalby PA. Engineering enzymes for biocatalysis. Recent Pat Biotechnol 2007;1:1–9.
[52] Yoshihiro H, Tadahiro O,Tooru K.. Mutated alkaline cellulase. US patent 7364891; 2008.
[53] Andersen KV, Schulein M, Christensen L, Damgaard B, Osten CVD . Cellulase variants. US patent 7993898 B2; 2011.
[54] Lavinge JA, Scott BR, Whissel M, Tomashek JJ. Family 6 cellulase with decreased inactivation by lignin. ; US patent 20100041100; 2010.
[55] Lavinge JA, Hill CMD, Tremblay A, St-Pierre P, Tomashek JJ. Cellulase variants with reduced inhibition by glucose. US patent 8012734; 2011.
[56] Okakura K, Yanai K. Cellulase tolerant to surfactants. US patent 7374921; 2008.
[57] St-Pierre P, Masri N, Fournier MC, White TC. Modified cellulases with increased thermostability, thermophilicity, and alkalophilicity. US patent 8101398; 2012.
[58] Wang H, Bao K. Neutral cellulase catalytic core and method of producing same. US patent 20060154843; 2009.
[59] Bower BS, Larenas EA, Mitchinson C. Exo-endo cellulase fusion protein. US patent 8097445; 2012.
[60] Gualfetti P, Mitchinson C, Phillips J. Variant EGIII-like cellulase compositions. US patent 7501272; 2009.
[61] Dashtban M, Maki M, Leung KT, Mao C, Qin W. Cellulase activities in biomass conversion: measurement methods and comparison. Crit Rev Biotechnol 2010;30:302–9.
[62] Shuangqi T, Zhenyu W, Ziluan F, Lili Z, Jichang W. Determination methods of cellulase activity. Afr J Biotechnol 2011;10:7122–5.
[63] Ng TK, Zeikus JGA. Continuous spectrophotometric assay for the determination of cellulase solubilizing activity. Anal Biochem 1980;103:42–50.
[64] Ng TK, Zeikus JG. Continuous spectrophotometric assay of microbial cellulase. US patent 4403032; 1983.
[65] Woodward J, Zachry GS. Immobilization of cellulase through its carbohydrate side chains – a rationale for its recovery and reuse. Enzyme Microbe Technol 1982;4:245–8.
[66] Ramos LP, Breuil C, Saddler JN. The use of enzyme recycling and the influence of sugar accumulation on cellulose hydrolysis by *Trichoderma cellulases*. Enzyme Microbe Technol 1993;15:19–25.

[67] Tu M, Chandra RP, Saddler JN. Evaluating the distribution of cellulases and the recycling of free cellulases during the hydrolysis of lignocellulosic substrates. Biotechnol Prog 2007;23:398–406.
[68] Wu JC, Ng KR, Chong J, Yang KJ, Lam XP, Nam CT, et al. Recovery of cellulases by adsorption/desorption using cation exchange resins. Korean J Chem Eng 2010;27:469–73.
[69] Singh A, Kumar PKR, Schügerl K. Adsorption and reuse of cellulases during saccharification of cellulosic materials. J Biotechnol 1991;18:205–12.
[70] Woodward J. Recovery and reuse of cellulase catalyst in an enzymatic cellulose hydrolysis process. US patent 4840904; 1989.
[71] Anish R, Rahman MS, Rao M. Application of cellulases from an alkalothermophilic *Thermomonospora* sp. in biopolishing of denims. Biotechnol Bioeng 2007;96:48–56.
[72] Clarkson K.A., Swanson B., Winetzky D. Cellulases for use in industrial applications. 2002; US patent 6451063.
[73] Emalfarb MA, Ben-Bassat A, Burlingame RP, Chernoglazov VM, Okounev ON, Olson PT, et al. Cellulase compositions and methods of use. US patent 5811381; 1998.
[74] Miettinen-Oinonen ASK, Elovainio MJ, Suominen PL. Cellulase composition for biofinishing cellulose-containing textile materials. US patent 5858767; 1999.
[75] Lenting HB Maria, Beckhoven RFWCV, Maurer K-He, Kottwitz B, Weiss A, Solingen PV. Detergents comprising cellulases. US patent US6767879 B2; 2004.
[76] Bjork N, Clarkson KA, Lad PJ, Weiss GL. Degradation resistant detergent compositions based on cellulase enzymes. US patent 5688290; 1997.
[77] Bettiol J-lP, Thoen CAJK. Alkaline detergent compositions comprising a specific cellulase. US patent 6187740; 2001.
[78] Baker RA, Wicker L. Current and potential applications of enzyme infusion in the food industry. Trends Food Sci Technol 1996;7:279–84.
[79] Nadeem MT, Butt MS, Anjum FM, Asgher M. Improving bread quality by carboxymethyl cellulase application. Int J Agric Biol 2009;11:727–30.
[80] Broeck HCVD, De Graaff LH, Visser J Van ooijen AJJ. Fungal cellulases. US patents 6190890; 2001.
[81] El-Sharnouby GA, Al-Eid MS, Al-Otaibi MM. Utilization of enzymes in the production of liquid sugar from dates. Afr J Biochem Res 2009;3:41–7.
[82] Haarasilta S, Pullinen T, Tammersalo-Karsten I, Vaisanen S, Franti H. Method of improving the production process of dry cereal products by enzyme addition. US patent 5176927; 1993.
[83] Jacobs JL, McAllan AB. Enzymes as silage additives. 1. Silage quality, digestion, digestibility and performance in growing cattle. Grass Forage Sci 1991;46:63–73.
[84] Krause M, Beauchemin KA, Rode LM, Farr BI, Nørgaard P. Fibrolytic enzyme treatment of barley grain and source of forage in high-grain diets fed to growing cattle. J Anim Sci 1998;76:2912–20.
[85] Virkki M, Apajalahti J, Visuri K. Method for treatment of fibrous crops with a modified cellulase to improve feed values storage and other properties. US patent 5948454; 1999.
[86] Charley RC. Enzyme system for silage preservation; 2011. WO2011050478 A1.
[87] Orgaz B, Kives J, Pedregosa AM, Monistrol IF, Laborda F, SanJosé C. Bacterial biofilm removal using fungal enzymes. Enzyme Microb Technol 2006;40:51–6.
[88] Wiatr CL. Application of cellulase to control industrial slime. US patent 4936994; 1990.
[89] Hernandez-Mena R, Friend PL. Enzyme treatment for industrial slime control. US patent 5238572; 1993.
[90] Kumar M. Enzymatic prevention prevention and control of biofilm. US patent 20080019956; 2008.
[91] Barnett CC, Manoj K, Whited GM. Enzymatic prevention prevention and control of biofilm. US patent 20110195059; 2011.
[92] Agbor VB, Cicek N, Sparling R, Berlin A, Levin DB. Biomass pretreatment: fundamentals toward application. Biotechnol Adv 2011;29:675–85.
[93] Larsen J, Petersen MO. Methods of processing ensiled biomass. US patent 8187848; 2012.
[94] Yanase S, Yamada R, Kaneko S, Noda H, Hasunuma T, Tanaka T, et al. Ethanol production from cellulosic materials using cellulase-expressing yeast. Biotechnol J 2010;5:449–55.
[95] Nakashima K, Yamaguchi K, Taniguchi N, Arai S, Yamada R, Katahira S, et al. Direct bioethanol production from cellulose by the combination of cellulase-displaying yeast and ionic liquid pretreatment. Green Chem 2011;13:2948–2953.
[96] Fatima H, El-Zaher A, Fadel M. Production of bioethanol via enzymatic saccharification of rice straw by cellulase produced by *Trichoderma reesei* under solid state fermentation. New York Sci J 2010;3:72–8.
[97] Singhvi M, Joshi D, Adsul M, Varma A, Gokhale D. D-(−)-Lactic acid production from cellobiose and cellulose by *Lactobacillus lactis* mutant RM2-24. Green Chem 2010;12:1106–9.
[98] Reddy HK, Srijana M, Reddy MD, Reddy G. Co culture fermentation of banana agro-waste to ethanol by cellulolytic thermophilic *Clostridium thermocellum* CT2. African J Biotechnol 2010;9:1926–34.
[99] Sangkharak K, Vangsirikul P, Janthachat S. Isolation of novel cellulase from agricultural soil and application for ethanol production. Int J Adv Biotechnol Res 2011;2:230–9.
[100] Ibrahim ASS, El-diwany AI. Isolation and identification of new cellulases producing thermophilic bacteria from an Egyptian hot spring and some properties of the crude enzyme. Aust J Basic Appl Sci 2007;1:473–8.
[101] Sahay H, Mahfooz S, Singh A, Singh S, Kaushik R, Saxena AK, et al. Exploration and characterization of agriculturally and industrially important haloalkaliphilic bacteria from environmental samples of hypersaline Sambhar lake, India. World J Microbiol Biotechnol 2012;28:3207–17.
[102] Gao J, Weng H, Zhu D, Yuan M, Guan F, Xi Y. Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. Bioresour Technol 2008;99:7623–9.
[103] Qin Y, He H, Li N, Ling M, Liang Z. Isolation and characterization of a thermostable cellulase-producing *Fusarium chlamydosporum*. World J Microbiol Biotechnol 2010;26:1991–7.
[104] Yin LJ, Huang PS, Lin HH. Isolation of cellulase-producing bacteria and characterization of the cellulase from the isolated bacterium *Cellulomonas* Sp. YJ5. J Agri Food Chem 2010;58:9833–7.
[105] Anand AAP, Vennison SJ, Sankar SG, Prabhu DIG, Vasan PT, Raghuraman T, et al. Isolation and characterization of bacteria from the gut of *Bombyx mori* that degrade cellulose, xylan, pectin and starch and their impact on digestion. J Insect Sci 2010;10:1–20.
[106] Nwodo-Chinedu S, Okochi VI, Smith HA, Omidij O. Isolation of cellulolytic microfungi involved in wood-waste decomposition: prospects for enzymatic hydrolysis of cellulosic wastes. Int J Biomed Health Sci 2005;1:41–52.
[107] Aydin YA, Aksoy ND. Isolation of cellulose producing bacteria from wastes of vinegar fermentation. In: World congress on engineering and computer science, vol. 1; 2009. p. 99–102.
[108] Wee Y-J, Kim S-Y, Yoon S-D, Ryu H-W. Isolation and characterization of a bacterial cellulose producing bacterium derived from the persimmon vinegar. Afr J Biotechnol 2011;10:16267–76.
[109] Baharuddin AS, Razak MNA, Hock LS, Ahmad MN, Abd-Aziz S, Rahman NAA, et al. Isolation and characterization of thermophilic cellulase-producing bacteria from empty fruit bunches-palm oil mill effluent compost. Am J Appl Sci 2010;7:56–62.
[110] Patel IB, Vaughn RH. Cellulolytic bacteria associated with sloughing spoilage of California ripe olives. Appl Microbiol 1973;25:62–9.
[111] Collmer A, Wilson DB. Cloning and expression of a *Thermomonospora* YX endocellulase gene in *E. coli*. Nat Biotech 1983;1:594–601.
[112] Li W, Zhang W-W, Yang M-M, Chen Y-L. Cloning of the thermostable cellulase gene from newly isolated *Bacillus subtilis* and its expression in *Escherichia coli*. Mol Biotechnol 2008;40:195–201.
[113] Wulff N, Carrer H, Pascholati S. Expression and purification of cellulase Xf818 from *Xylella fastidiosa* in *Escherichia coli*. Curr Microbiol 2006;53:198–203.
[114] Reinhold-Hurek B, Hurek T, Claeyssens M, van Montagu M. Cloning, expression in *Escherichia coli*, and characterization of cellulolytic enzymes of *Azoarcus* sp. a root-invading diazotroph. J Bacteriol 1993;175:7056–65.
[115] Barros ME, Thomson JA. Cloning and expression in *Escherichia coli* of a cellulase gene from *Ruminococcus flavefaciens*. J Bacteriol 1987;169:1760–2.
[116] Sumitomo N, Ozaki K, Hitomi J, Kawaminami S, Kobayashi T, Kawai S, et al. Application of the upstream region of a *Bacillus endoglucanase* gene to high-level expression of foreign genes in *Bacillus subtilis*. Biosci Biotechnol Biochem 1995;59:2172–5.
[117] Ghangas GS, Wilson DB. Expression of a *Thermomonospora fusca* cellulase gene in *Streptomyces lividans* and *Bacillus subtilis*. Appl Environ Microbiol 1987;53:1470–5.
[118] van der Vlugt-Bergmans CJB, van Ooyen AJJ. Expression cloning in *Kluyveromyces lactis*. Biotechnol Techniq 1999;13:87–92.
[119] Thongekkaew J, Ikeda H, Masaki K, Iefuji H. An acidic and thermostable carboxymethyl cellulase from the yeast *Cryptococcus* sp. S-2: purification, characterization and improvement of its recombinant enzyme production by high cell-density fermentation of *Pichia pastoris*. Protein Expr Purif 2008;60:140–6.
[120] Boer H, Teeri TT, Koivula A. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. Biotechnol Bioeng 2000;69:486–94.
[121] Godbole S, Decker SR, Nieves RA, Adney WS, Vinzant TB, Baker JO, et al. Cloning and expression of *Trichoderma reesei* cellobiohydrolase I in *Pichia pastoris*. Biotechnol Prog 1999;15:828–33.
[122] Okada H, Tada K, Sekiya T, Yokoyama K, Takahashi A, Tohda H, et al. Molecular characterization and heterologous expression of the gene encoding a low-molecular-mass endoglucanase from *Trichoderma reesei* QM9414. Appl Environ Microbiol 1998;64:555–63.
[123] Okada H, Sekiya T, Yokoyama K, Tohda H, Kumagai H, Morikawa Y. Efficient secretion of *Trichoderma reesei* cellobiohydrolase II in *Schizosaccharomyces pombe* and characterization of its products. Appl Microbiol Biotechnol 1998;49:301–8.
[124] Park C, Chang C, Ryu DY. Expression and high-level secretion of *Trichoderma reesei* endoglucanase I in *Yarrowia lipolytica*. Appl Biochem Biotechnol 2000;87:1–15.
[125] Liu D, Zhang R, Yang X, Zhang Z, Song S, Miao Y, et al. Characterization of a thermostable β-glucosidase from *Aspergillus fumigatus* Z5, and its functional expression in *Pichia pastoris* X-33. Microb Cell Fact 2012;11:25, http://dx.doi.org/10.1186/1475-2859-11-25.
[126] Skory CD, Freer SN, Bothast RJ. Expression and secretion of the *Candida wickerhamii* extracellular β-glucosidase gene, bglB, in *Saccharomyces cerevisiae*. Curr Genet 1996;30:417–22.
[127] Kawai R, Yoshida M, Tani T, Igarashi K, Ohira T, Nagasawa H, et al. Production and characterization of recombinant *Phanerochaete chrysosporium* beta-glucosidase in the methylotrophic yeast *Pichia pastoris*. Biosci Biotechnol Biochem 2003;67:1–7.

[128] Jones BE, Van der Kleij AW, Van solingen P, Weyler W. Cellulase producing actinomycetes, cellulase produced there from and method of producing same. US patent 6566112; 2003.
[129] Cheng K-J, Liu J-H, Tsai C-F., Hsu Y-C. Cellulase enzymes. US patent 6428996; 2002.
[130] Brumm P. Thermostable cellulase and methods of use. US patent 7510857; 2009.
[131] Jones BE, Grant WD, Heaphy S, Grant S. *Bacillus* 029cel cellulase. US patent 7604974; 2009.
[132] Solingen PV. Alkaline cellulase and method of producing same. US patent 5856165; 1999.
[133] Li X-L, Ljungdahl LG, Chen H. Cellulases and coding sequences. US patent 6190189; 2001.
[134] Jones BE, Grant WD, Heaphy S, Grant S. *Bacillus* BagCel cellulase. US patent 7892807; 2011.
[135] Jorgensen PL, Schulein M, Hansen C. Enzyme exhibiting cellulase activity. US patent 5916796; 1999.
[136] Wicher KB, Holst OP, Hachem YA, Karlsson EMN, Hreggvidsson GO. Thermostable cellulase. US patent 6812018; 2004.
[137] Mathur EJ, Lam DE. Carboxymethyl cellulase from *Thermotoga maritima*.; US patent 5962258; 1999.
[138] Lastick SM, Tucker MP, Grohmann K. Cloning of cellulase genes from *Acidothermus cellulolyticus*. US patent 5514584; 1996.
[139] Broeck HCVD, De Graaff LH, Visser J., Van ooijen AJJ. Fungal cellulases.; US patents 6190890; 2001.
[140] Farrington GK, Anderson P, Bergquist P, Daniels R, Gibbs MD, Morgan H, et al. Compositions and methods for treating cellulose containing fabrics using truncated cellulase enzyme compositions. US patent 6294366; 2001.
[141] Fowler T, Barnett CC, Shoemaker S. Cloning and amplification of the β-glucosidase gene of *Trichoderma reesei*. US patent 6022725; 2000.
[142] Ward M, Clarkson KA, Larenas EA, Lorch JD, Weiss GL. Sequence encoding endoglucanase III cellulase. US patent 5475105; 1995.